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Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92

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ABSTRACT

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Aims: To investigate aggregation and adhesiveness of *Lactobacillus acidophilus* M92 to porcine ileal epithelial cells *in vitro*, and the influence of cell surface proteins on autoaggregation and adhesiveness of this strain.

Methods and Results: *Lactobacillus acidophilus* M92 exhibits a strong autoaggregating phenotype and manifests a high degree of hydrophobicity determined by microbial adhesion to xylene. Aggregation and hydrophobicity were abolished upon exposure of the cells to pronase and pepsin. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of cell surface proteins revealed the presence of potential surface layer (S-layer) proteins, approximated at 45 kDa, in *L. acidophilus* M92. The relationship between autoaggregation and adhesiveness to intestinal tissue was investigated by observing the adhesiveness of *L. acidophilus* M92 to porcine ileal epithelial cells. Removal of the S-layer proteins by extraction with 5 mol l^{-1} LiCl reduced autoaggregation and *in vitro* adhesion of this strain.

Conclusions: These results demonstrate that there is relationship between autoaggregation and adhesiveness ability of *L. acidophilus* M92, mediated by proteinaceous components on the cell surface.

Significance and Impact of the Study: This investigation has shown that *L. acidophilus* M92 has the ability to establish in the human gastrointestinal tract, which is an important determinant in the choice of probiotic strains.

Keywords: adhesion, aggregation, hydrophobicity, *Lactobacillus acidophilus*, probiotic properties, surface layer proteins.

INTRODUCTION

Adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract, preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem (Pedersen and Tannock 1989; Freter 1992; Alander *et al.* 1997). Difficulties involved in studying bacterial adhesion *in vivo*, especially in humans, have led to the development of *in vitro* model systems for the preliminary selection of potentially adherent strains (Mäyrä-Mäkinen *et al.* 1983, Conway and Kjellberg 1989; Kimoto *et al.* 1999).

Bacterial adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesins (usually proteins) and complementary receptors (Freter 1992; Rojas and Conway 1996; Pérez *et al.* 1998). Autoaggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cells, and coaggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms (Reid *et al.* 1988; Boris *et al.* 1997; Del Re *et al.* 2000). Physicochemical characteristics of the cell surface such as hydrophobicity may affect autoaggregation and adhesion of bacteria to different surfaces (Wadström *et al.* 1987; Pérez *et al.* 1998; Del Re *et al.* 2000). The proteinaceous nature of some surface components has been demonstrated, and surface layer (S-layer) proteins detected in some *Lactobacillus* strains may be involved in adherence (Schneitz *et al.* 1993; Mukai and Arihara 1994).

Lactobacillus acidophilus is one of the major species of this genus found in human and animal intestines. When present

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in sufficient numbers, as probiotics, the lactobacilli are believed to be able to create a healthy equilibrium between beneficial and potentially harmful microflora in the gut (Tannock 1999; Šušković *et al.* 2001). The potential probiotic strain *L. acidophilus* M92 has shown the ability to resist digestion processes in the gastrointestinal tract and is bile resistant (Kos *et al.* 2000; Šušković *et al.* 2000). Furthermore, *in vitro* studies have shown that *L. acidophilus* M92 can assimilate cholesterol in the presence of bile, so it is postulated that this strain might help in lowering serum cholesterol *in vivo* (Kos 2001).

The aim of this study was to investigate the aggregation abilities and adhesive properties of *L. acidophilus* M92 to porcine ileal epithelial cells *in vitro,* which were used because of the similarity of porcine and human intestinal tracts. The role of cell surface proteins on autoaggregation and adhesiveness of *L. acidophilus* M92 was also determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus acidophilus M92, *Lactobacillus plantarum* L4, *Enterococcus faecium* L3, *Escherichia coli* 3014 and *Salmonella* serotype Typhimurium from the culture collection of the Department of Biochemical Engineering, University of Zagreb and the standard strain *L. acidophilus* ATCC 4356 were used in this study. All lactic acid bacteria were stored at −70°C in de Man Rogosa Sharpe (MRS) broth (Difco, Detroit, MI, USA) with 30% glycerol. *Escherichia coli* 3014 and *S.* Typhimurium were maintained on nutrient agar (Difco) slopes at 4°C. Before experimental use, cultures were subcultured twice in MRS or in nutrient broth (Difco).

Autoaggregation and coaggregation assays

Autoaggregation assays were performed according to Del Re *et al.* (2000) with certain modifications. Bacteria were grown for 18 h at 37°C with MRS solid or liquid medium. The cells were harvested by centrifugation at 5000 *g* for 15 min, washed twice and resuspended in their culture supernatant fluid or in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU ml^{-1}. Cell suspensions (4 ml) were mixed by vortexing for 10 s and autoaggregation was determined during 5 h of incubation at room temperature. Every hour 0·1 ml of the upper suspension was transferred to another tube with 3·9 ml of PBS and the absorbance (A) was measured at 600 nm. The autoaggregation percentage is expressed as: $1-(A_t/A_0) \times 100$, where A_t represents the absorbance at time $t = 1$, 2, 3, 4 or 5 h and A_0 the absorbance at $t = 0$.

The method for preparing the cell suspensions for coaggregation was the same as that for autoaggregation assay. Equal volumes (2 ml) of each cell suspension were mixed together in pairs by vortexing for 10 s. Control tubes were set up at the same time, containing 4 ml of each bacterial suspension on its own. The absorbance (A) at 600 nm of the suspensions were measured after mixing and after 5 h of incubation at room temperature. Samples were taken in the same way as in the autoaggregation assay. The percentage of coaggregation was calculated using the equation of Handley *et al.* (1987):

$$\text{Coaggregation } (\%) = \frac{((A\text{x} + A\text{y})/2) - A(\text{x} + \text{y})}{A\text{x} + A\text{y}/2} \times 100$$

where x and y represent each of the two strains in the control tubes, and (x + y) the mixture.

Microbial adhesion to solvents

Microbial adhesion to solvents (MATS) was measured according to the method of Rosenberg *et al.* (1980) with some modifications (Crow and Gopal, 1995; Bellon-Fontaine *et al.* 1996). Bacteria were harvested in the stationary phase by centrifugation at 5000 *g* for 15 min, washed twice, and resuspended in 0·1 mol l^{-1} KNO_3 (pH 6·2) to approximately 10^8 CFU ml^{-1}. The absorbance of the cell suspension was measured at 600 nm (A_0). One millilitre of solvent was added to 3 ml of cell suspension. After a 10-min preincubation at room temperature, the two-phase system was mixed by vortexing for 2-min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance at 600 nm (A_1) was measured. The percentage of bacterial adhesion to solvent was calculated as $(1-A_1/A_0) \times 100$.

Three different solvents were tested in this study: xylene (Kemika, Zagreb, Croatia), which is an apolar solvent; chloroform (Kemika), a monopolar and acidic solvent; and ethyl acetate (Kemika), a monopolar and basic solvent. Only bacterial adhesion to xylene reflects cell surface hydrophobicity or hydrophilicity. The values of MATS obtained with the two other solvents, chloroform and ethyl acetate, were regarded as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively (Bellon-Fontaine *et al.* 1996).

In vitro test for adhesion of *L. acidophilus* M92 to pig intestinal epithelium

The adhesion test was peformed using the method of Mäyrä-Mäkinen *et al.* (1983), with modifications. Ileal samples were collected from 6-month-old Landras pigs. The tissues were held in PBS at 4°C for 30 min to loosen surface mucus, and then washed three times with PBS. The adhesion test was performed by incubating tissue samples (1 cm^2) in bacterial

suspensions (10^9 cells ml^{-1} PBS) at 37°C for 30 min. Samples of ileum were fixed in 10% formalin, dehydrated by increasing concentrations of ethanol, and embedded in paraffin. Serial sections (5 μm) were cut, mounted on standard microscope slides and stained for identification of Gram-positive and Gram-negative bacteria, according to Brown and Brenn (Švob 1974). Slides were examined and photographed using a Nikon Mikrophot-FXA light microscope (Nikon, Tokyo, Japan).

Enzymic and chemical treatments of bacterial cells

Cultures were harvested by centrifugation (5000 *g*, 15 min), washed twice with distilled water and resuspended in the appropriate buffer. Cell suspensions, prepared as described above, were subjected to different pH values of pronase, pepsin, metaperiodate and LiCl. All chemicals were obtained from Sigma (St Louis, MO, USA). The effect of different pH values on hydrophobicity and autoaggregation ability of *L. acidophilus* M92 was assayed in 0·1 mol l^{-1} citrate/phosphate buffer, pH 2·8 and 0·1 mol l^{-1} acetate buffer, pH 4·5. Pronase treatment (1 g l^{-1}, 30 min at 37°C) was carried out in PBS, pH 7·2, pepsin treatment (0·5 g l^{-1}, 30 min) in 0·1 mol l^{-1} citrate/phosphate buffer, pH 2·8 and metaperiodate treatment (10 g l^{-1}, 30 min) in 0·1 mol l^{-1} acetate buffer, pH 4·5. The S-layer proteins of *L. acidophilus* M92 and *L. acidophilus* ATCC 4356 were removed by extraction with 5 mol l^{-1} LiCl (30 min at 37°C). Subsequently, bacterial cell suspensions were washed twice and resuspended in PBS for MATS, aggregation and adhesion assays.

SDS-PAGE of surface proteins from bacterial cells

Untreated bacteria or cells which had been extracted with 5 mol l^{-1} LiCl, as described above, were resuspended in 1% SDS for 30 min at 37°C, for the isolation of surface proteins. After centrifugation (9000 *g*, 5 min), the supernatant was analysed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970), using 10% polyacrylamide gels. Protein bands were visualized by staining the gels with Coomassie brilliant blue. Low molecular weight markers from 14·4 to 97·0 kDa (α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin and phosphorylase b) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

RESULTS

Autoaggregation, coaggregation and adhesiveness of *L. acidophilus* M92

The sedimentation rate of *L. acidophilus* M92 was measured over a period of 5 h. Results showed that the strain exhibited a strong autoaggregating phenotype. Better growth of the bacterium on MRS broth than on MRS agar could be the reason for slightly better autoaggregation of cells grown on MRS broth. The observed autoaggregation could be related to cell surface component, because it was not lost after washing and suspending of the cells in PBS (Fig. 1).

Coaggregation of *L. acidophilus* M92 with two other potential probiotic strains (*L. plantarum* L4, *Ent. faecium* L3) and two enteropathogens (*S.* Typhimurium, *E. coli*) was also examined (Table 1). Results are expressed as the percentage reduction after 5 h in the absorbance of a mixed suspension compared with the individual suspension. *Lactobacillus acidophilus* M92 demonstrated marked coaggregation with *Ent. faecium* L3 (Fig. 2), *E. coli* and *S.* Typhimurium (Table 1).

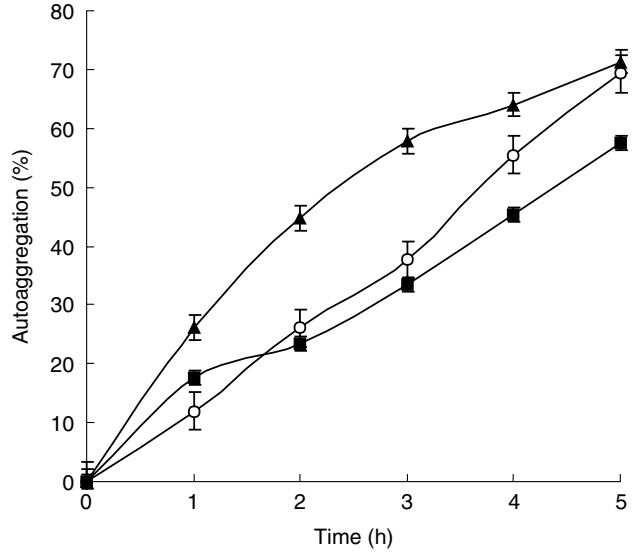

Fig. 1 Comparison of the autoaggregation ability of *Lactobacillus acidophilus* M92 cells resuspended in PBS (pH 7·2) after grown on MRS agar (■) and in MRS broth (▲) or resuspended in their own culture supernatant fluid (○). Error bars represent standard deviations of the mean values of results from three replicate experiments

Table 1 Coaggregation ability of *Lactobacillus acidophilus* M92 after 5 h incubation at room temperature in PBS (pH 7·2)

Bacteria	Coaggregation with *L. acidophilus* M92 (%)
Lactobacillus plantarum L4	4·36 (±1·81)
Enterococcus faecium L3	19·46 (±2·63)
Escherichia coli 3014	15·11 (±2·07)
Salmonella serotype Typhimurium	15·70 (±2·98)

Mean (±standard deviation) of results from three separate experiments.

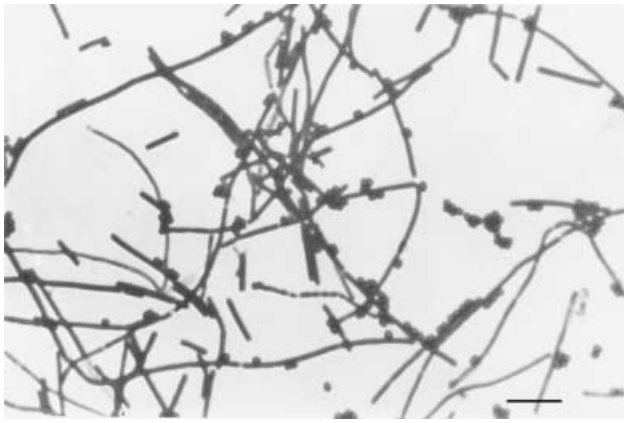

Fig. 2 Coaggregation between *Lactobacillus acidophilus* M92 and *Enterococcus faecium* L3 incubated in PBS (pH 7·2). Magnification, ×1000. Bar represents 10 μm

The adhesiveness of *L. acidophilus* M92 to the intestinal tissue was also investigated. Microscopic examinations showed that this species strongly adhered to porcine ileal epithelial cells (Fig. 3).

Influence of cell surface properties on autoaggregation and adhesion of *L. acidophilus* M92

The MATS method was used to evaluate the hydrophobic/hydrophilic cell surface properties of *L. acidophilus* M92 and compare them with the cell surface properties of two other probiotic bacteria. The results indicated that *L. acidophilus* M92 was hydrophobic, whereas *L. plantarum* L4 and *Ent. faecium* L3 were fully hydrophilic (Table 2). Bacterial adhesion to chloroform and ethyl acetate was tested to assess the Lewis acid–base characteristics of the bacterial cell surfaces. All strains showed stronger affinity for chloroform, which is an acidic solvent and electron acceptor, than for ethyl acetate, which is a basic solvent and electron donor (Table 2). *Lactobacillus acidophilus* M92 was further subjected to different treatments to characterize the cell surface components responsible for its hydrophobicity and autoaggregation ability. Table 3 shows that the hydrophobicity of *L. acidophilus* M92 slightly decreased

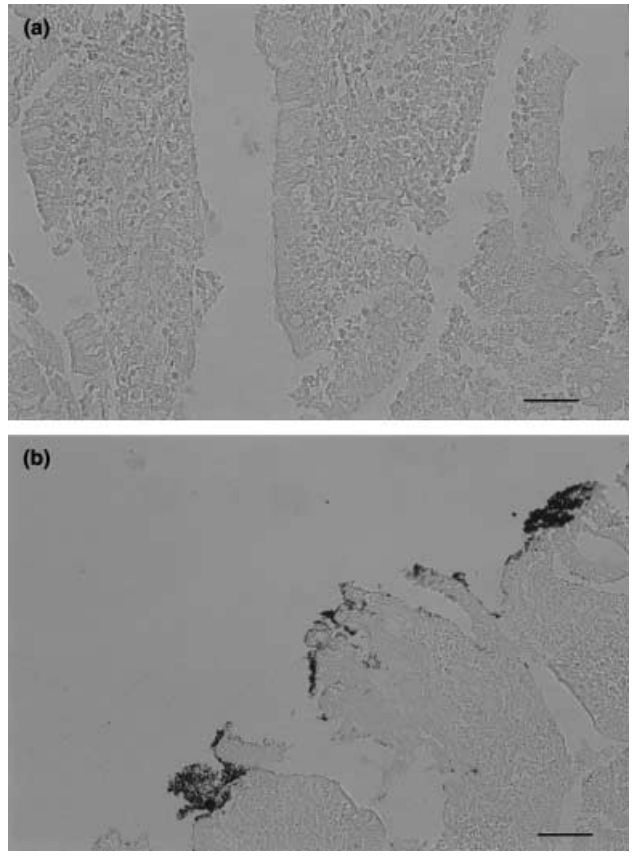

Fig. 3 Adhesion test with *Lactobacillus acidophilus* M92: intestinal epithelium of the pig before (a) and after treatment with suspension of *L. acidophilus* M92 cells (b). Magnification, ×200. Bars represent 60 μm

at pH 2·8, but pepsin treatment eliminated this property. The same effect on hydrophobicity was found with pronase. Moreover, autoaggregation ability was moderately reduced when *L. acidophilus* M92 was subjected to pronase and pepsin activity. By oxidizing cell surface carbohydrates with metaperiodate, the cell surface hydrophobicity was slightly affected, but autoaggregation of *L. acidophilus* M92 was not significantly affected. When the surface proteins of *L. acidophilus* M92 were extracted and analysed by SDS-PAGE (Fig. 4) the dominant protein band of *L. acidophilus* M92 had a molecular mass of approximately 45 kDa. After

Table 2 Adhesion of *Lactobacillus acidophilus* M92, *L. plantarum* L4 and *Enterococcus faecium* L3 to xylene, chloroform and ethyl acetate

	Adhesion (%)		
Bacteria	Xylene	Chloroform	Ethyl acetate
Lactobacillus acidophilus M92	70·96 (±1·63)	36·06 (±1·28)	0
Lactobacillus plantarum L4	6·52 (±1·43)	47·03 (±1·84)	18·32 (±0·98)
Enterococcus faecium L3	0	61·21 (±2·02)	0

Mean (±standard deviation) of results from three separate experiments.

Table 3 Effect of different treatments on cell surface hydrophobicity and autoaggregation ability of *Lactobacillus acidophilus* M92

Treatment	Cell surface hydrophobicity (%)	Autoaggregation (%)
pH 7·2	70·96 (±2·11)[a]	71·30 (±2·08)[a]
pH 4·5	73·59 (±1·33)[a]	68·89 (±1·84)[a]
pH 2·8	55·85 (±1·47)[b]	62·14 (±1·27)[b]
Pronase	0	58·12 (±1·84)[c]
Pepsin	0	54·52 (±2·17)[c]
Metaperiodate	64·84 (±1·34)[c]	67·17 (±2·78)[a]

Values in parentheses represent the standard deviation. Mean values ($n = 3$) with no common superscript letters differ significantly ($P < 0·05$).

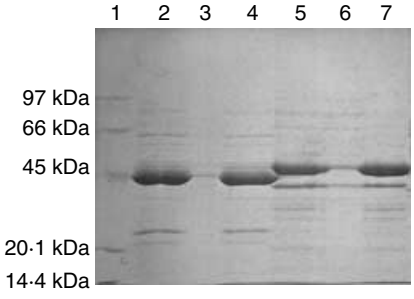

Fig. 4 SDS-PAGE of cell surface proteins from *Lactobacillus acidophilus* ATCC 4356 and *L. acidophilus* M92 before and after extraction with 5 mol l^{-1} LiCl; lanes 2, 3 and 4, *L. acidophilus* ATCC 4356 cell surface proteins from untreated cells (lanes 2 and 4) and from cells extracted with 5 mol l^{-1} LiCl (lane 3); lanes 5, 6 and 7, *L. acidophilus* M92 cell surface proteins from untreated cells (lanes 5 and 7) and from cells extracted with 5 mol l^{-1} LiCl (lane 6); lane 1, low molecular weight protein standards

extraction of bacterial cells with 5 mol l^{-1} LiCl, more than 90% of S-layer proteins from both *L. acidophilus* M92 and the control strain *L. acidophilus* ATCC 4356 were removed. After S-layer removal, these two strains were tested for autoaggregation. In both cases, removal of S-layer proteins reduced their autoaggregation ability (Fig. 5). When the contribution of these proteins in adhesiveness of *L. acidophilus* M92 to pig intestinal epithelium was examined, the results suggested that these proteins could be involved in adhesion processes, because of weaker adhesiveness of LiCl-treated bacterial cells in relation to untreated cells (Fig. 6).

DISCUSSION

The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains used as probiotics. Cell adhesion is a multistep process involving contact of the bacterial cell

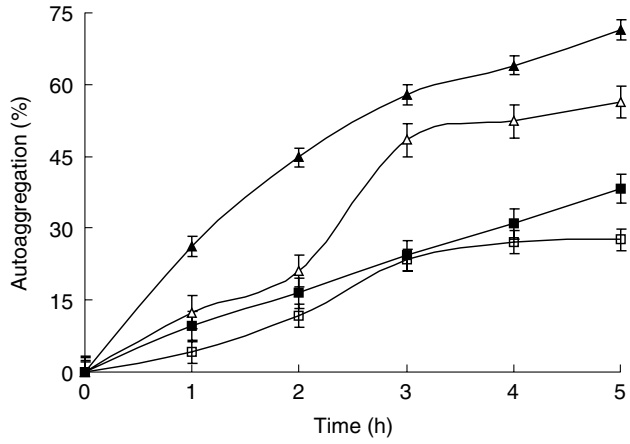

Fig. 5 Comparison of the autoaggregation ability of *Lactobacillus acidophilus* M92 and *L. acidophilus* ATCC 4356 before (▲ M92, ■ ATCC 4356) and after removal of their surface layer proteins with 5 mol l^{-1} LiCl (△ M92, □ ATCC 4356). Error bars represent standard deviations of the mean values of results from three replicate experiments

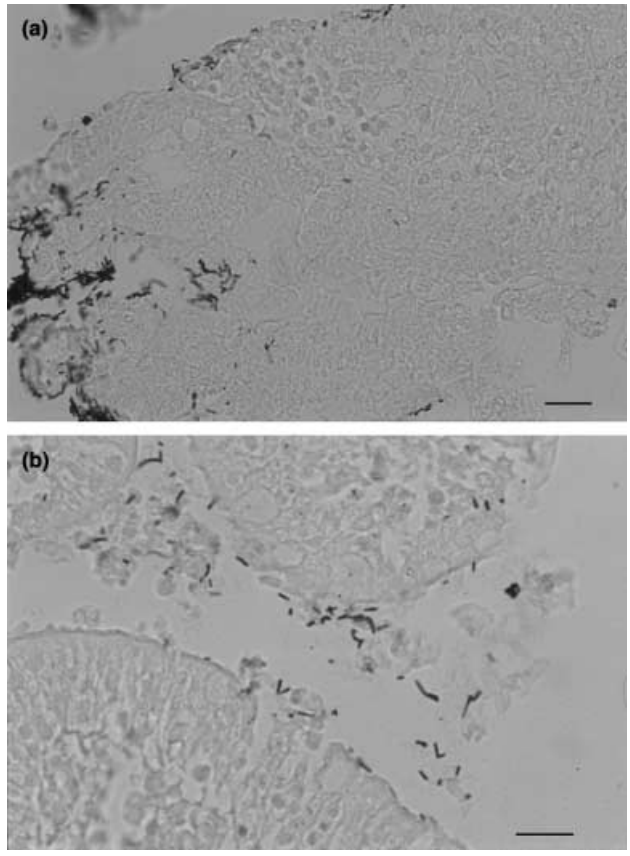

Fig. 6 Adhesion of *Lactobacillus acidophilus* M92 to the intestinal epithelium cells of the pig before (a) and after treatment of bacterial cells with 5 mol l^{-1} LiCl (b). Magnification, ×600. Bars represent 20 μm

membrane and interacting surfaces. Several workers have investigated the composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells (Green and Klaenhammer 1994; Pelletier *et al.* 1997; Pérez *et al.* 1998; Del Re *et al.* 2000). In most cases, aggregation ability is related to cell adherence properties (Vandevoorde *et al.* 1992; Boris *et al.* 1997; Del Re *et al.* 2000). Therefore, the potential probiotic strain *L. acidophilus* M92 was examined for its autoaggregation ability and coaggregation with some lactic acid bacteria and potential pathogenic bacteria. Broth-grown and agar-grown cells of *L. acidophilus* M92, suspended in PBS (pH 7·2) were compared for their autoaggregation ability, because the method of culture has been recognized as a factor that may affect bacterial aggregation (Reid *et al.* 1992; Spencer and Chesson 1994). To avoid the possibility of removing extracellular components, which may have been concerned with autoaggregation, broth-grown cells were additionally examined for autoaggregation ability resuspended in their own culture supernatant fluid. *Lactobacillus acidophilus* M92 showed a strong autoaggregating phenotype which was not lost after washing and suspending of the cells in PBS. To quantify interbacterial adherence, a coaggregation assay was developed, which established coaggregation between *L. acidophilus* M92 and two other potential probiotic strains, particularly *Ent. faecium* L3, which could increase their colonization potential if they were to be used in mixed culture as probiotics. Furthermore, it has been suggested that inhibitor producing lactic acid bacteria, which coaggregate with pathogens, may constitute an important host defence mechanism against infection in the urogenital tract (Reid *et al.* 1988). Also, a similar protective mechanism could operate in the gastrointestinal tract (Spencer and Chesson 1994), and in our previous work, *L. acidophilus* M92 showed antagonistic activity against enteropathogenic *E. coli* and *S.* Typhimurium (Šušković *et al.* 1997). Coaggregation with potentially gut pathogens could therefore contribute to the probiotic properties ascribed to lactic acid bacteria.

In order to gain information on the structural properties of the cell surface of *L. acidophilus* M92 that are responsible for aggregation and adhesion, its hydrophobicity/hydrophilicity was compared with two other lactic acid bacteria (*L. plantarum* L4 and *Ent. faecium* L3). The fact that a high percentage of *L. acidophilus* M92 cells adhered to xylene, an apolar solvent, demonstrated hydrophobic cell surface of this strain. However, *L. plantarum* L4 and *Ent. faecium* L3 showed more hydrophilic cell surface properties, and strong affinities to chloroform, which means they are strong electron donors. Many previous studies on the physicochemistry of microbial cell surfaces have shown that the presence of (glyco-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides (Green and Klaenhammer 1994; Rojas and Conway 1996; Pelletier *et al.* 1997). It is known that only pronase- and pepsin-sensitive surface molecules are responsible for cell surface hydrophobicity in bacteria. In this study, the autoaggregation ability of *L. acidophilus* M92 was also reduced by proteolytic treatment, while metaperiodate did not much affect either hydrophobicity, or autoaggregation (Table 3). As bacterial cells subjected to proteolytic attack weaken their autoaggregation ability, it is reasonable to consider proteins as mediators in the aggregation process. The SDS-PAGE of cell surface proteins of *L. acidophilus* M92 revealed the presence of S-layer proteins, of an approximate molecular mass of 45 kDa. The S-layer proteins form a crystalline layer around many bacterial species, amounting to 15–20% of the total cellular protein content, apparent molecular weights of 40–200 kDa (Pouwels *et al.* 1998; Sára and Sleytr 2000). It has been proposed that these proteins are involved in cell protection and surface recognition, and that they could be potential mediators in the initial steps involved in adhesion (Schneitz *et al.* 1993; Green and Klaenhammer 1994; Mukai and Arihara 1994). To assess the potential contribution of these proteins to autoaggregation and adherence, bacterial cells were extracted with 5 mol l^{-1} LiCl to remove S-layer proteins. The results showed that these proteins are important for autoaggregation in *L. acidophilus* M92. Furthermore, adherence was markedly different between non-treated bacterial cells and cells treated with 5 mol l^{-1} LiCl (Fig. 6a, b). The reduction of adherence after removing S-layer proteins from the cell surface suggests that these proteins promoted the adhesiveness of *L. acidophilus* M92. However, the role of S-layer proteins in the adhesion of *L. acidophilus* to intestinal epithelial cells is not clearly understood. While Green and Klaenhammer (1994) observed that carbohydrate and protein substances on the cell surface other than those in the S-layer are responsible for adhesion, Schneitz *et al.* (1993) stated that the opposite was true. Furthermore, Mukai and Arihara (1994) found that glycoproteins in the S-layer on the surface of *L. acidophilus* JCM 1132 bind to lectins on the intestinal epithelial cells.

In conclusion, our findings indicate that there is a relationship between autoaggregation and adhesiveness of *L. acidophilus* M92 that are mediated by proteinaceous components on the cell surface.

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